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## Identification of nitric oxide synthase in human and bovine oviduct

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# Identification of nitric oxide synthase in human and bovine oviduct

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Nitric oxide synthase (NOS) is responsible for the biological production of nitric oxide (NO) in several organs. NOS activity has also been localized in the reproductive tract, although direct evidence for its presence in the human or bovine oviduct is still lacking. In the present study, four different techniques were used to identify the presence of NOS activity in human ( $n = 11$ ) and bovine ( $n = 9$ ) oviduct: (i) conversion of [<sup>3</sup>H]-L-arginine to [<sup>3</sup>H]-L-citrulline; (ii) production of nitrite/nitrate (NO<sub>2</sub>/NO<sub>3</sub>; stable NO metabolites); (iii) identification of NADPH-diaphorase activity; and (iv) immunostaining with antiserum to endothelial NOS. Cytosolic extracts from human ampullary segments of the Fallopian tube, obtained from post-partum patients ( $n = 4$ ), converted [<sup>3</sup>H]-L-arginine to [<sup>3</sup>H]-L-citrulline ( $21.0 \pm 8.8$  fmol/mg protein/min). This conversion rate was significantly ( $P < 0.05$ ) reduced in the presence of either EDTA or *N*-monomethyl-L-arginine monoacetate (L-NMMA), an inhibitor of NOS activity. When bovine ( $n = 3$ ) ampullary segments were incubated for 36 h in Hanks' balanced salt solution, the concentration of NO<sub>2</sub>/NO<sub>3</sub> in the medium was increased ( $P < 0.05$ ) if segments were pretreated with lipopolysaccharide (LPS; an inducer of inducible NOS), but not after treatment with LPS + L-NMMA. Additionally, epithelial cells cultured from ampullary segments showed positive staining both for NADPH-diaphorase activity and with antiserum to endothelial NOS. The results of the present study provide direct evidence for the presence of both the Ca<sup>2+</sup>-dependent constitutive form of NOS, as well as the inducible form of NOS activity in human and bovine oviduct. Since the oviduct plays a key role in the reproductive process, it is possible that the two forms of NOS may be involved in the physiological regulation of oviduct function.

**Key words:** bovine/human/nitric oxide/nitric oxide synthase/oviduct

## Introduction

The oviduct plays a key role in the reproductive process by regulating the transport of gametes and embryos, as well as by providing a specific micro-environment for both the fertilization process and the primary stages of embryonic development (Sayegh and Mastroianni, 1991). Smooth muscle cell contraction and relaxation, ciliary beats of the oviduct epithelial cells and regulation of the synthesis of oviduct-derived factors are essentially involved in this process. Recently we reported that endothelin, a potent contracting factor (Yanagisawa *et al.*, 1991), is synthesized by bovine oviduct epithelial cells *in vitro* (Rosselli *et al.*, 1994a) and is able to induce contraction of bovine oviduct segments (Rosselli *et al.*, 1994b). We also demonstrated that the contractile effects of endothelin-1 on bovine oviducts are enhanced by the presence of *N*-monomethyl-L-arginine monoacetate (L-NMMA), an inhibitor of nitric oxide (NO) synthesis. These findings provided the first indirect evidence for endogenous synthesis of NO within the oviduct (Rosselli *et al.*, 1994b).

NO is synthesized by NO synthase (NOS), which utilizes the semi-essential amino acid L-arginine as a substrate. The

biological events that are mediated by NO can be prevented by L-arginine analogues, including L-NMMA, which compete with L-arginine for NOS and inhibit NO synthesis (Moncada *et al.*, 1991). NOS is an enzyme known to exist in several tissue and cell types and three isoforms of the enzyme have been identified. Two isoforms of constitutive NOS, brain NOS [(bNOS) and endothelial NOS (eNOS)] are responsible for the continuous basal release of NO. Constitutive NOS (cNOS) activity requires Ca<sup>2+</sup>/calmodulin and can be inhibited by Ca<sup>2+</sup> chelators such as EDTA. Furthermore, cNOS activity can be stimulated by hormones such as oestrogens, mechanical factors, and agonists such as endothelin (Förstermann *et al.*, 1991; Moncada *et al.*, 1991; Weiner *et al.*, 1994). The third isoform is an inducible NOS (iNOS) that can be expressed in response to cytokines and lipopolysaccharide (LPS). Under specific conditions, such as immune reactions, iNOS generates excessive amounts of NO even in the absence of Ca<sup>2+</sup> (Morris and Billiar, 1994).

Since NO is a labile substance with a short half-life, its direct measurement has proven to be difficult. However, several techniques are now available to evaluate NOS activity or NO synthesis in cultured cells, organ cultures and tissue extracts. In both biological fluids and culture media NO rapidly

decomposes/metabolizes into  $\text{NO}_2$  and  $\text{NO}_3$ . Furthermore, stoichiometric and biochemical studies have shown that this  $\text{NO}_2/\text{NO}_3$  is derived from NO generated through NOS and can be used as an index of NO production in different cell and organ cultures *in vitro* (Marletta *et al.*, 1988; Ochoa *et al.*, 1991; Havel and Marletta, 1994).

Another method used for analysing NOS activity is the direct measurement of L-citrulline, a metabolite of L-arginine that is generated simultaneously with the release of each molecule of NO (Bredt and Snyder, 1990). Additionally, histochemical markers for the localization of NOS have also been established. For example, immunostaining of cells with antiserum to NOS has been widely used to confirm the presence of NOS in various tissues and reproductive organs (Burnett *et al.*, 1995). Furthermore, recent observations indicate that NOS has NADPH-diaphorase like activity and that cells expressing NOS also contain abundant NADPH-diaphorase activity (Dawson *et al.*, 1991). NADPH-diaphorase has been shown to co-localize with cytochrome P450 reductase (Kemp *et al.*, 1988), an enzyme that has sequence homology with various isoforms of NOS, requires NADPH and flavin, and is involved in electron transfer (Bredt *et al.*, 1991). Indeed, using NADPH-diaphorase as a marker, several studies have now demonstrated the presence of NOS activity in different tissues including the reproductive organs (Burnett *et al.*, 1995). These findings suggest that immunostaining with antiserum to NOS and detection of NADPH-diaphorase activity are useful tools for the histochemical identification and/or localization of NOS in cultured cells. Hence, by using four different techniques, we aimed to identify the presence of cNOS and iNOS activity in the human and bovine oviduct.

## Materials and methods

Ampullary segments of human oviducts (0.1–0.17 g) were obtained 1–3 days post-partum from patients undergoing sterilization ( $n = 11$ ). Bovine oviducts ( $n = 9$ ) from young non-pregnant cyclic cows were obtained from the local abattoir and the fimbrial, ampullary and isthmus segments were dissected and used for the study.

Oviducts were placed immediately into cold Hanks' balanced salt solution (HBSS; Sigma, Buch, Switzerland) containing 100  $\mu\text{g}$  streptomycin/ml, 100 IU/ml penicillin, 0.025  $\mu\text{g}$ /ml amphotericin B (Gibco Laboratories, Basel, Switzerland). For each experiment, oviducts were cleaned of the surrounding tissue and repeatedly washed with HBSS.

### Epithelial cell culture

Bovine epithelial cells from the ampullary segment of the oviduct were isolated and cultured using the method described previously by Rosselli *et al.* (1994a). Briefly, the lumen of the oviduct was gently perfused with 10 ml HBSS. The epithelial cells were then mechanically dislodged from the oviduct by rapidly flushing it three times with 20 ml calcium-magnesium-free HBSS. Epithelial cell layers were dislodged easily and were in the form of sheets. Isolated epithelial cells were then suspended in HBSS and washed twice by centrifuging at 300 g for 10 min. The final cell pellet was suspended in 4 ml HBSS, then layered over 5 ml Percoll (1.07 g/ml HBSS) and centrifuged at 1000 g for 20 min. The cells present at the interface were collected, washed twice with HBSS and then resuspended in complete culture medium (Ham's F10 culture media, 100  $\mu\text{g}$ /ml

streptomycin, 100 IU/ml penicillin, 0.025 g/ml amphotericin B, 10% fetal calf serum (FCS; Gibco) and plated in 12-well culture plates. A similar procedure was used for preparation of human oviduct epithelial cells. The cells were allowed to attach and grow in complete culture medium for 6–7 days and were then used for the immuno-histochemical localization of NOS. Epithelial cell purity was ascertained by criteria as previously reported (Rosselli *et al.*, 1994a); characteristic cell morphology, growth pattern and positive immunostaining with anti-cytokeratin antibodies CK1 (Dako, Diagnostic AG, Zug, Switzerland).

### Conversion of arginine to citrulline

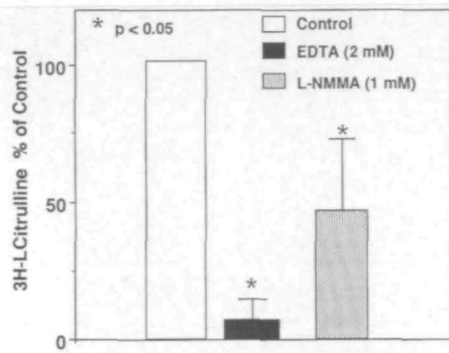
NOS activity was measured in human ampullary segments ( $n = 4$ ) and in the fimbrial, isthmus, and ampullary segments of bovine oviducts ( $n = 3$ ). Tissues were homogenized with 1 ml of cold Tris-HCl (50 mM) containing 2 mM EDTA. The homogenates were centrifuged for 15 min at 8000 g and 4°C and the supernatants were stored at  $-20^\circ\text{C}$  until further analysis. NOS activity was measured by the method of Bredt and Snyder (1990). Briefly, 25  $\mu\text{l}$  aliquots of the supernatant ( $4 \pm 1.7$  mg protein/ml) were incubated with 100  $\mu\text{l}$  of reaction buffer containing 1  $\mu\text{Ci}/\text{ml}$  (16 pmol/ml) [ $^3\text{H}$ ]-L-arginine (specific activity 63 Ci/mmol; Amersham, Zurich, Switzerland), 50 mM HEPES (pH 7.4), 1 mM NADPH, 12.5 mM  $\text{CaCl}_2$ , 12 mM flavine adenine dinucleotide (FAD) and 1 mM dithiothreitol. After incubation for 20 min at room temperature, the reaction was terminated by adding 2 ml of termination buffer (20 mM HEPES, pH 5.5; 2 mM EDTA). The samples were then applied to 0.6 ml columns of Dowex AG50WX 8 (Na<sup>+</sup> form; BioRad, Zurich, Switzerland) to remove unreacted [ $^3\text{H}$ ]-L-arginine. [ $^3\text{H}$ ]-L-citrulline in the column eluate was quantified by counting the radioactivity in a  $\beta$ -scintillation counter. Some enzyme assays were conducted in the presence of either EDTA (2 mM) or 1 mM L-NMMA (Boehringer Mannheim, Rotkreuz, Switzerland). Protein concentration in the enzyme extracts was measured using the procedure of Lowry *et al.* (1951).

### Nitrite/nitrate assay

Bovine ampullary segments (0.13–0.17 g) were incubated for 36 h in: (i) HBSS; (ii) 7.3 mM L-arginine; (iii) 7.3 mM L-arginine + 10  $\mu\text{g}/\text{ml}$  LPS (Sigma); (iv) 7.3 mM L-arginine + 10  $\mu\text{g}/\text{ml}$  LPS + 1 mM L-NMMA. The  $\text{NO}_2/\text{NO}_3$  concentration in the incubation media was measured using Greiss reagent after conversion of nitrate to nitrite with nitrate reductase (Cayman's nitrite/nitrate assay kit; Alexis Corporation, Läufelfingen, Switzerland) and as described previously (Green *et al.*, 1982; Rosselli *et al.*, 1994c). Amounts of  $\text{NO}_2$  in the media were estimated from a standard curve obtained by enzymatic conversion of  $\text{NaNO}_3$  (0–32  $\mu\text{mol}/\text{l}$ ). Similar experiments were conducted with human ampullary segments ( $n = 4$ ). Ampullary segments (0.15–0.17 g) were incubated in: (i) 7.3 mM L-arginine; (ii) 7.3 mM L-arginine + 10  $\mu\text{g}/\text{ml}$  LPS; or (iii) 7.3 mM L-arginine + 10  $\mu\text{g}/\text{ml}$  LPS + 1 mM L-NMMA.

### NADPH-diaphorase staining

The NOS activity was also confirmed in cultured human ( $n = 3$ ) and bovine oviduct epithelial cells ( $n = 3$ ) by identifying histochemically the presence of NADPH-diaphorase activity. Primary cultures of epithelial cells (as cultured above) were washed three times with phosphate-buffered saline (PBS), and then fixed at room temperature for 20 min with 4% paraformaldehyde in PBS. Fixed cells were washed three times with PBS and incubated for 1 h at  $37^\circ\text{C}$  in a staining buffer (50 mM Tris-HCl pH 7.4, 1 mM NADPH, 0.5 mM nitroblue tetrazolium, 0.25% Triton-X 100). The cells were subsequently washed with PBS, mounted with aquamount (Merck Dietikon, Switzerland) and examined under a microscope.



**Figure 1.** Bar graph showing nitric oxide synthase (NOS) activity measured in cytosolic extracts of ampullary segments of human oviduct. NOS activity was assayed by measuring the formation of [ $^3\text{H}$ ]-L-citrulline from [ $^3\text{H}$ ]-L-arginine in the presence or absence (control) of 2 mM EDTA or 1 mM *N*-monomethyl-L-arginine monoacetate (L-NMMA). Values are given as a percentage of control values (fmol [ $^3\text{H}$ ]-L-citrulline formed per min per mg protein). Each bar represents mean  $\pm$  SEM from four separate experiments. \*Significantly different from control values.

#### Immunostaining with endothelial NOS antiserum

Primary cultures of human and bovine oviduct epithelial cells grown for 6–7 days were washed three times with PBS and then fixed at room temperature with 4% paraformaldehyde for 20 min. The fixed cells were sequentially washed and treated as follows: washed three times with PBS, incubated for 5 min at room temperature in 3% hydrogen peroxide, washed three times with PBS and incubated in PBS containing 10% bovine serum albumin (BSA; Sigma) for 1 h. The cells were subsequently washed with PBS containing 1% BSA and incubated overnight with eNOS antiserum (rabbit antiserum to eNOS, bovine, Alexis Corporation) at a dilution of 1:200 in PBS 1% BSA. The second antibody used was biotinylated anti-mouse, anti-rabbit immunoglobulin and staining was performed with peroxidase (LSAB (R) 2 Kit, peroxidase; Dako). Epithelial cells incubated overnight with PBS 1% BSA and treated with the second antibody system were used as negative controls.

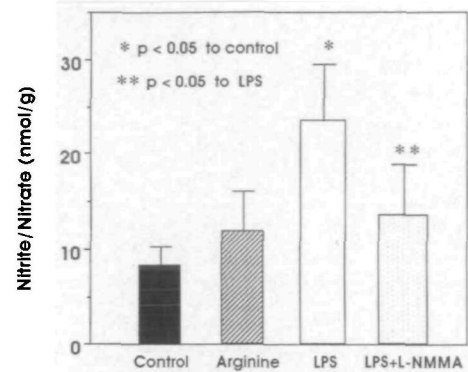
#### Statistical analysis

Data obtained for the conversion of [ $^3\text{H}$ ]-L-arginine to [ $^3\text{H}$ ]-L-citrulline and production of  $\text{NO}_2/\text{NO}_3$  are presented as mean  $\pm$  SEM. Statistical analysis was performed with analysis of variance (ANOVA) and paired or unpaired *t*-test, as appropriate. A value of  $P < 0.05$  was considered to be statistically significant.

## Results

#### Conversion of arginine to citrulline

Incubation of cytosolic extracts obtained from the ampullary segments of human oviduct with [ $^3\text{H}$ ]-L-arginine for 20 min resulted in the generation of [ $^3\text{H}$ ]-L-citrulline, suggesting that NOS activity is present in the oviduct. The average value of this activity was  $21.0 \pm 8.8$  fmol/min/mg protein ( $n = 4$ ), and large inter-subject variation in NOS activity was observed. Incubation of these extracts in the presence of EDTA, which inhibits eNOS activity by chelating the essential co-factor  $\text{Ca}^{2+}$ , blocked the formation of [ $^3\text{H}$ ]-L-citrulline from [ $^3\text{H}$ ]-L-arginine (Figure 1). Moreover L-NMMA, which inhibits NO synthesis by competing with L-arginine, significantly reduced the formation of  $^3\text{H}$  L-citrulline from [ $^3\text{H}$ ]-L-arginine (Figure



**Figure 2.** Bar graph depicting nitric oxide synthase (NOS) activity in bovine oviducts. Production of NO was measured by analysing the concentrations of nitrite/nitrate in the incubation medium in which ampullary segments were incubated for 36 h in: (i) Hanks' balanced salt solution (HBSS; control); (ii) 7.3 mM arginine (Arginine); (iii) 7.3 mM L-arginine + 10  $\mu\text{g}/\text{ml}$  lipopolysaccharide (LPS); (iv) 7.3 mM L-arginine + 10  $\mu\text{g}/\text{ml}$  LPS + 1 mM L-NMMA (LPS + L-NMMA). Each bar represents the mean  $\pm$  SEM from three experiments. \*Significantly different ( $P < 0.05$ ); \*\*significantly different ( $P < 0.01$ ).

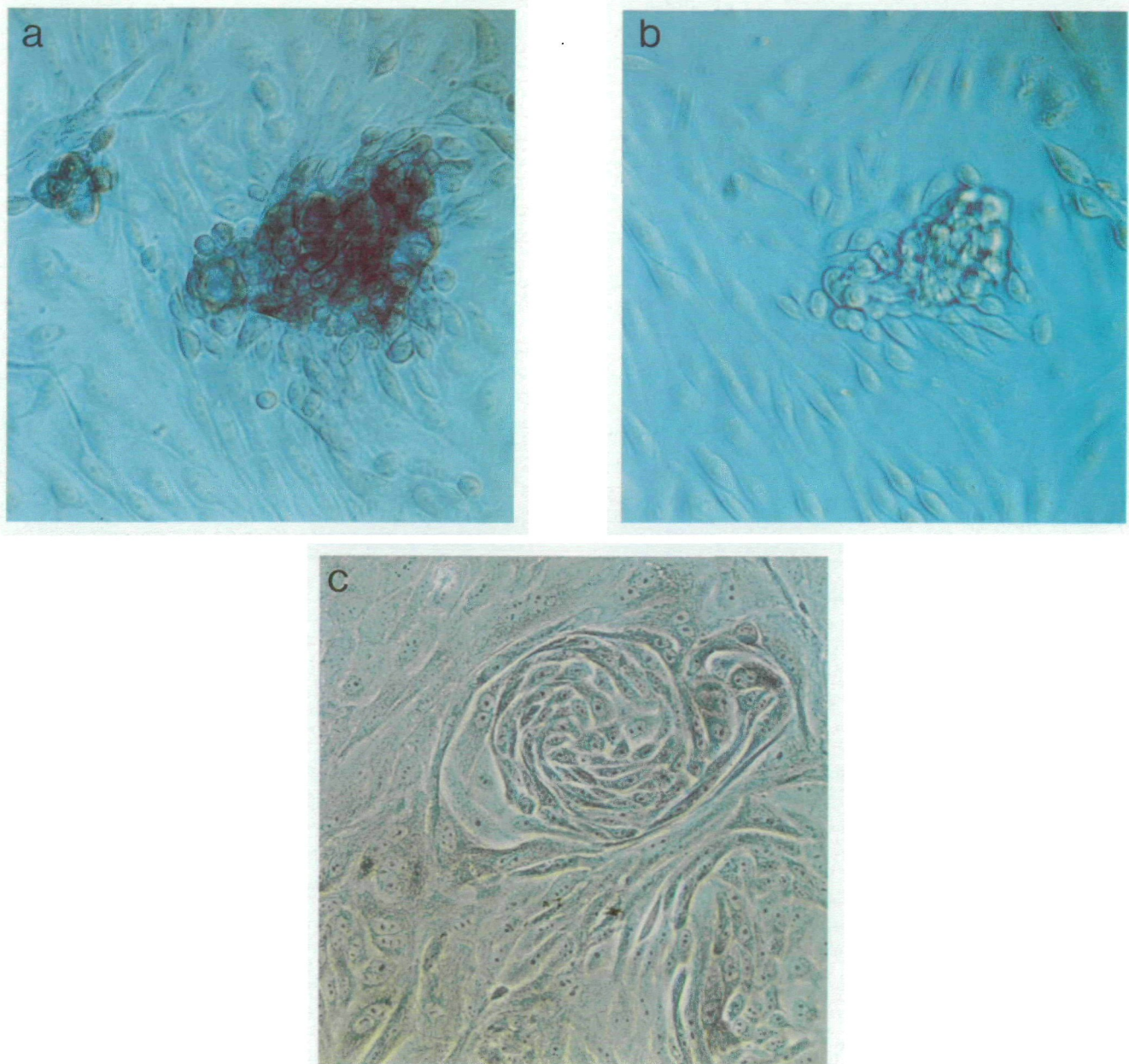
1). When compared with untreated controls, NOS activity in the presence of EDTA or L-NMMA was significantly reduced from  $21 \pm 8.8$  fmol/min/mg protein to  $0.55 \pm 0.5$  fmol/min/mg protein ( $P < 0.05$ ;  $8.0 \pm 8.0\%$  of control values) and to  $3.0 \pm 1.5$  fmol/min/mg protein ( $P < 0.05$ ;  $28 \pm 21\%$  of control values) respectively (Figure 1). Similar results were also obtained with the bovine ampullary segments.

Cytosolic fractions extracted from three different segments of the bovine oviduct, i.e. fimbria, ampulla and isthmus were also able to metabolize [ $^3\text{H}$ ]-L-arginine to [ $^3\text{H}$ ]-L-citrulline. The NOS activity in the three different areas of the oviduct, the fimbria, ampulla and the isthmus did not vary significantly and was  $10.7 \pm 1.5$ ,  $11.7 \pm 3.7$  and  $11.9 \pm 5.4$  fmol/min/mg protein respectively.

#### Nitrate/nitrite assay

The presence of NOS activity in human and bovine oviducts was confirmed by analysing the generation of  $\text{NO}_2/\text{NO}_3$  (metabolites of NO) by ampullary segments. When compared with control segments ( $n = 3$ ) incubated in HBSS, a significant increase in  $\text{NO}_2/\text{NO}_3$  concentrations was observed in segments incubated with 7.3 mM L-arginine + 10  $\mu\text{g}/\text{ml}$  LPS ( $P < 0.05$ ; Figure 2). Furthermore, no increase in NO synthesis ( $\text{NO}_2/\text{NO}_3$  concentrations) in response to 7 mM L-arginine + 10  $\mu\text{g}/\text{ml}$  LPS was observed in segments incubated with LPS in the presence of L-NMMA (Figure 2). To confirm the presence of iNOS in human oviducts, similar experiments were conducted with human ampullary segments ( $n = 4$ ). When compared with human ampullary segments incubated in the presence of 7.3 mM L-arginine, pretreatment with 10  $\mu\text{g}/\text{ml}$  LPS enhanced the generation of NO from  $15.2 \pm 1.2$  to  $22.5 \pm 1.7$  nmol/g tissue. Moreover, the LPS-increased  $\text{NO}_2/\text{NO}_3$  values were reduced to  $13.8 \pm 1.3$  nmol/g tissue when L-NMMA was present in the medium.





**Figure 3.** (a) Representative photomicrographs showing the histochemical staining for NADPH-diaphorase in cultured human oviduct epithelial cells. Cultured human oviduct epithelial cells were incubated in the presence of NADPH and nitroblue tetrazolium (original magnification  $\times 200$ ). (b) Cells stained in the absence of NADPH (negative control). (c) Similar results were obtained from cells obtained from three separate human oviducts and three different bovine oviducts (original magnification  $\times 100$ ).

#### **NADPH-diaphorase and anti-eNOS staining**

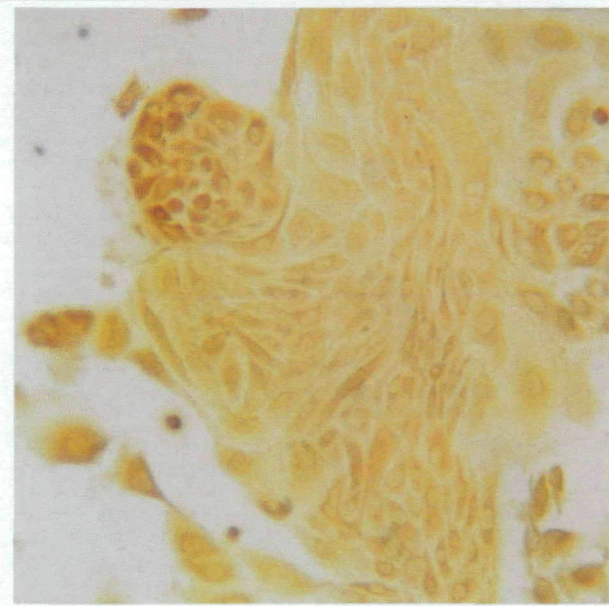
The above findings provide direct biochemical evidence of the presence of NOS activity within human and bovine oviducts. Presence of NOS activity in the epithelial lining of the oviduct was further confirmed immuno-histochemically by immunostaining cultured human ( $n = 3$ ) and bovine ( $n = 3$ ) epithelial cells using antiserum to eNOS and by establishing the presence of NADPH-diaphorase, an enzyme which has been shown to co-localize with NOS. Morphological and histochemical characterization of epithelial cells cultured from human and bovine oviducts suggests that the preparation was not contaminated with other cell types. Human and bovine epithelial cells showed intense positive staining for NADPH-diaphorase in the presence, but not in the absence, of NADPH

(Figure 3). The staining was not homogeneous and several areas of intense staining were observed. Positive staining was also observed in human and bovine oviduct epithelial cells treated with antiserum to eNOS (Figure 4), however no staining was observed in negative control cells treated with second antibody alone.

#### **Discussion**

The oviduct contributes significantly to the reproductive process by controlling the transport of gametes and embryos. Contraction and relaxation of smooth muscle within the oviduct, as well as the ciliary beats of oviduct epithelial cells, are major factors in promoting and regulating this transport.





**Figure 4.** Representative photomicrographs showing cultured human oviduct epithelial cells incubated with antiserum to endothelial nitric oxide synthase (eNOS) (1:200 dilution) and stained with peroxidase. The epithelial cells (original magnification  $\times 200$ ) showed intense positive staining. Negative control cells treated with phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and second antibody alone did not stain. Similar results were observed from cultured epithelial cells obtained from three different human oviducts as well as from three different bovine oviducts.

Several autocrine and paracrine factors may be responsible for the regulation of this contractile mechanism within the oviduct. We have previously shown that bovine oviduct epithelial cells are capable of generating endothelin, a potent contractile peptide, and that the contractile effects of endothelin-1 on bovine oviducts are enhanced by the presence of L-NAME, an inhibitor of NO synthesis (Rosselli *et al.*, 1994b). These findings led us to speculate that within the oviduct there is endogenous synthesis of NO, and we hypothesized that the basal generation of NO may be important in maintaining normal tone within the oviduct. The occurrence of endogenous NO synthesis within the oviduct was confirmed in the present study by measuring both the conversion of [ $^3$ H]-L-arginine to [ $^3$ H]-L-citrulline and the generation of NO<sub>2</sub>/NO<sub>3</sub> as biochemical markers. By immunostaining with eNOS antiserum and histochemically for NADPH-diaphorase, we provided direct evidence of the presence of cNOS and iNOS activity in both human and bovine oviducts.

NO has been identified in the male (Burnett *et al.*, 1995) and female reproductive tract (Ellman *et al.*, 1993; Yallampally *et al.*, 1994; Bryant *et al.*, 1995; Telfer *et al.*, 1995). In uterine tissues it seems to play an important role during pregnancy (Yallampally *et al.*, 1994); whereas in the ovary it is potentially involved in the ovulatory process (Ellman *et al.*, 1993). NO has also been shown to regulate the sexual behaviour in female rats (Mani *et al.*, 1994) and steroids, including 17  $\beta$ -oestradiol, have been shown to regulate NOS activity (Rosselli *et al.*, 1994c; Weiner *et al.*, 1994). This NOS activity is present in the Fallopian tube of the rat and is regulated during the oestrus

cycle (Bryant *et al.*, 1995). Based on these findings, it is possible that NO may play a multi-functional role within the reproductive tract. In this regard, we recently provided the first indirect evidence for the presence of NOS in the bovine oviduct (Rosselli *et al.*, 1994a). Additionally, we showed that increased NO synthesis decreases both sperm motility and viability, and hypothesized that under pathological conditions this could lead to infertility (Rosselli *et al.*, 1995). NO generated by the endothelial lining of the vascular bed is known to induce relaxation of the underlying smooth muscle cells (Moncada *et al.*, 1991). Moreover, NO can also regulate the contractile state of smooth muscle cells in other organs, including urinary bladder (Persson *et al.*, 1991) and uterus (Yallampally *et al.*, 1994). Under physiological conditions, continuous generation of low amounts of NO (basal synthesis), specifically via calcium-dependent eNOS, is responsible for regulating smooth muscle cell contraction and relaxation, and maintaining normal tone of the conduits such as blood vessels. Hence, it is likely that basal synthesis of NO within the oviduct may also regulate the tone and contractility of smooth muscle cells.

Our findings that, under non-stimulated conditions, NOS activity was present in the human and bovine oviducts, and that this activity was inhibited by EDTA, a chelator of calcium, suggest that there is basal synthesis of NO within the oviduct and that the possible source of this NO is the calcium-dependent constitutive NOS. This notion is further supported by our observations of positive immunostaining of cultured epithelial cells with antiserum to eNOS, and of intense staining for NADPH-diaphorase, an enzyme which co-localizes with eNOS (Dawson *et al.*, 1991).

We also observed that segments of oviduct incubated for 36 h were able to generate a significant amount of NO<sub>2</sub>/NO<sub>3</sub> under L-arginine-free conditions, and that this increased marginally when the medium was supplemented with L-arginine. This implies either that the oviduct segments have a substantial amount of endogenous L-arginine or are capable of generating L-arginine, thus providing the substrate for basal NO generation. Moreover, the fact that NOS activity was observed in various segments of the bovine oviduct suggests that it is distributed throughout the organ. The distribution of the NOS activity in the human oviduct is, however not known, and needs to be investigated. Furthermore our histochemical observation, that cultured epithelial cells possessed areas of intense staining for NADPH-diaphorase and immunostaining for eNOS, suggests that NOS enzyme activity may be dependent on the differentiation of the cells. This possibility also needs to be further investigated.

Based on our finding, it could be argued that basal generation of NO by oviduct epithelial cells may contribute significantly to the contraction and relaxation of the oviduct and hence actively participate in the reproductive process. Moreover, impairment of NO synthesis under pathological conditions may lead to abnormal contractility or tone of the oviduct and consequently contribute to infertility.

In addition to eNOS, several tissues have been shown to contain inducible NOS (Morris and Billiar, 1994). In contrast to eNOS, iNOS does not generate NO under basal conditions

and is calcium-independent. Furthermore, toxins, cytokines and LPS generated during localized infections can activate iNOS and result in the generation of large amounts of NO (Morris and Billiar, 1994). Excessive NO, in turn, can have cytotoxic and cytostatic effects on the invading organism and the surrounding tissue directly or in combination with free radicals (Beckman et al., 1990). Our observation that LPS treatment of human as well as bovine oviduct ampullary segments increased NO synthesis ( $\text{NO}_2/\text{NO}_3$ ) in the absence, but not in the presence, of L-NAME suggests that this increase is due to activation of iNOS, and provides the first evidence for the presence of iNOS within the oviduct.

Due to the unavailability of non-pathological human oviductal tissue from women in normal menstrual cycles, NOS activity was measured in oviductal tissue obtained 1–3 days post-partum. Since hormones associated with pregnancy have been shown to modulate NOS activity in various tissues (Weiner et al., 1994; Yallampalli et al., 1994; Bryant et al., 1995), it is possible that NOS activity in post-partum oviducts may not reflect the true physiological activity. In contrast, although the presence of hormones may have influenced the quantitative estimates of NOS activity measured biochemically in fresh tissues, it is unlikely to have influenced the qualitative presence of the NOS enzyme determined histochemically in cultured oviduct epithelial cells. The immuno-histochemical localization of eNOS was conducted in epithelial cells cultured for several days in hormone-free medium, allowing sufficient time for the wash-out of hormones associated with pregnancy. Thus, positive immunostaining of cultured epithelial cells with eNOS antiserum and the presence of NADPH-diaphorase activity better reflect the presence of NOS activity under physiological conditions. However, it is also possible that other factors in post-partum oviducts may have influenced the quantitative estimates of NOS activity. This remains a limiting factor of the present study. Further studies using non-pathological oviductal tissue from women with normal menstrual cycles need to be conducted.

In conclusion, using four different techniques for the determination of NOS activity, we provide the first direct evidence for the presence of both basal/constitutive and inducible NOS in human and bovine oviducts. Our findings suggest that the basal synthesis of NO within the oviduct may play a vital role as a local relaxing factor and contribute to the rhythmic contraction of the oviduct, essential for regulating the transport of gametes and embryos. The presence of iNOS activity within the oviduct suggests that large amounts of NO generated via activation of iNOS under pathological conditions such as infection or endometriosis may impair its normal contractility, affect the transport of gametes and embryos, provoke sperm toxicity, and consequently result in infertility or ectopic pregnancy.

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